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Applicant: Dahm *et al.*

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For: *METHOD FOR THE QUANTITATIVE DETERMINATION OF TUMOR CELLS IN A BODY FLUID AND TEST KITS SUITABLE THEREOF*

Art Unit: 1655

Examiner: Zitomer, S.

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Kelly Fischer

MARKED UP SPECIFICATION AND CLAIMS (37 C.F.R. § 1.121)

IN THE SPECIFICATION:

Please amend the specification as follows:

At page 1, line 1, please insert:

—This application is the National Stage of International Application No. PCT/EP99/00716, filed February 3, 1999. Benefit of priority under 35 U.S.C. §365(b) to German Application No. 198 04 372.4, filed February 4, 1998 is claimed herein.—

Please amend the paragraph on page 7, line 27 to page 8, line 26, as follows:

The subsequent amplification can be carried out, for example, with DNA polymerase, for example by the polymerase chain reaction (PCR) (see, for example, U.S. Patent Nos. 4,683,195; 4,683,202; 4,965,188) or, preferably, with an RNA polymerase by, for example, isothermal nucleic acid sequence-based amplification (NASBA). Specific oligonucleotide primers derived from the nucleic acid to be amplified are required for the amplification in each case. It is possible in the present invention to use any sequence section of the cDNA coding for the catalytic subunit of telomerase for synthesizing the oligonucleotide primers. The oligonucleotide primers are preferably about 20 to

about 40, preferably about 25 to 35, nucleotides long. The amplification product is generally about 100 to about 2000 bases, preferably about 200 to about 1500 bases, in particular about 450 to about 550 bases, long. The following oligonucleotide primers, which have been derived from the sequence shown in Fig. 1, are particularly preferred for the novel method:
5′ CTACCGGAAG AGTGTCTGGA GCAAGTTGCA AAGC 3′ (hTRT1) (SEQ ID NO. 1), and/or
5′ GGCATACCGA CGCACGCAGT ACGTGTTCTG 3′ (hTRT2) (SEQ ID. NO. 2), where hTRT1 and/or hTRT2 may, where appropriate, additionally comprise a promoter sequence for an RNA polymerase. The oligonucleotide primer hTRT1 corresponds to the 5′ primer and hTRT2 corresponds to the 3′ primer. The amplification product is 513 bp long. The primers may, for example, be prepared synthetically using the triester methods (Matteucci et al., (1981), J. Am. Chem. Soc., 103, 3185-3191). The DNA polymerase which can be used is, for example, a non-thermostable DNA polymerase such as T4 DNA polymerase, T7 DNA polymerase, E. coli polymerase I or the Klenow fragment of E. coli or, preferably, a thermostable DNA polymerase such as Taq polymerase (see, for example, U.S. Patent No. 4,889,818).

Please amend the paragraph on page 14, lines 27-39 as follows:

As internal positive control of the method and of the sample to be investigated it is possible additionally to amplify and detect a nucleic acid which generally always occurs in a body fluid. Examples of suitable nucleic acids are the mRNA coding for β-globin or for glyceraldehyde-phosphate dehydrogenase (GAPDH) (see, for example, GB 2 260 811) which always occur in the cells of the body fluid. Suitable oligonucleotide primers for human β-globin mRNA are, for example, primers with the sequences:
5′ ACCCAGAGGT TCTTTGAGTC 3′ (Glob 1) (SEQ ID. NO. 3) and
5′ TCTGATAGGC AGCCTGCACT 3′ (Glob 2) (SEQ ID. NO. 4)

Please amend the paragraph on page 15, lines 1-22 as follows:

Further internal positive controls of the method and of the sample to be investigated can additionally be cell-specific nucleic acids, such as β-actin mRNA, with the primers (Nakajima-Iiji, S., Hamada, H., Reddy, P., Kakanuga, T. (1985): Molecular structure of the cytoplasmatic β-actin gene: Interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 82, 6133-7):

5′ GATGATGATATCGCCGCGCTCGTC 3′ (Act 1) (SEQ ID. NO. 5)

5′ CTCAAACATGATCTGGGTCATCTTC 3′ (Act 2) (SEQ ID. NO. 6)

or T-cell-specific nucleic acids, such as the mRNA of the T-cell receptor, with the primers (Toyonaga, B., Yoshikai, Y., Vadasz, V., Chin, B., Mak, T.W. (1985): Organization and sequences of the diversity, joining, and constant region of the human T-cell receptor β chain. Proc Natl Acad Sci USA 82, 8624-8):

5′ GAGGTCGCTGTGTTTGAGCCATCAGAAG 3′ (TCR 1) (SEQ ID. NO. 7)

5′ GATCTCATAGAGGATGGTGGCAGACAG 3′ (TCR 2) (SEQ ID. NO. 8)

Please amend the paragraph on page 26, line 25 to page 27, line 3 as follows:

The present invention further relates to the oligonucleotide primers with the sequence

5′ CTACCGGAAG AGTGTCTGGA GCAAGTTGCA AAGC 3′ (hTRT1)

(SEQ ID. NO. 1) and/or

5′ GGCATACCGA CGCACGCAGT ACGTGTTCTG 3′ (hTRT2) (SEQ ID. NO. 2),

where hTRT1 and/or hTRT2 may, where appropriate, additionally comprise a promoter sequence for an RNA polymerase;

and an oligonucleotide with the sequence

5′ CGTTCTGGCT CCCACGACGT AGTC 3′ (hTRT o) (SEQ ID. NO. 9)

and the corresponding reverse complementary sequences of the oligonucleotide for detecting the amplified "antisense" RNA.

Please amend the paragraph on page 27, lines 4-20 as follows:

The invention additionally relates to a kit for quantifying tumor cells in a body fluid, for example blood, urine or else stool, exudates or transudates from body cavities, especially peripheral blood, comprising

(a) oligonucleotide primer pair for specific amplification of telomerase-encoding nucleic acid, where the oligonucleotide primer pair preferably has the following sequences:

5' CTACCGGAAG AGTGTCTGGA GCAAGTTGCA AAGC 3' (hTRT1) (SEQ ID. NO. 1) and/or

5' GGCATACCGA CGCACGCAGT ACGTGTTCTG 3' (hTRT2) (SEQ. ID. NO. 2), where hTRT1 and/or hTRT2 may, where appropriate, additionally comprise a promoter sequence for an RNA polymerase.

Please amend the paragraph on page 28, lines 27-24 as follows:

Fig. 1 shows the sequence described by Nakamura et al., (SEQ ID. NO. 10) encoding the catalytic subunit of human telomerase, of 4015 base pairs (bp), and the position of the designed oligonucleotide primers or the oligonucleotide probe (hTRT o) (SEQ ID. NO. 9; underlined): 5' primer hTRT1 (position 1780-1813) (SEQ ID. NO. 1), 3' primer hTRT2 (position 2261-2290) (SEQ ID. NO. 2) with an amplification product of 513 base pairs (bp) and the probe hTRT o (position 1964-1987) (SEQ ID. NO. 9).

Please amend the paragraph on page 35, lines 14-28 as follows:

The two oligonucleotide primers:

5' CTACCGGAAG AGTGTCTGGA GCAAGTTGCA AAGC 3' (hTRT1) (SEQ. ID. NO. 1) and

5' GGCATACCGA CGCACGCAGT ACGTGTTCTG 3' (hTRT2) (SEQ ID. NO. 2) were designed in accordance with the sequence, published by Nakamura et al., coding for the catalytic subunit of human telomerase (Nakamura et al. (1997). Science 277: 955-9) (Fig. 1 and SEQ ID. NO. 10) and synthesized using an Applied Biosystem 380A synthesizer. The specificity of the hTRT1 and hTRT2 primers was checked by computer-assisted analysis of homology on the nucleic

acid sequences in the GenBank, EMBL, DDBJ and PDB databases using BLASTIN 1.4.9 MP (Altschul, S.F. et al. (1990), J Mol Biol 215: 403-410].

Please amend the paragraph on page 37, line 3 to page 38, line 18 as follows:

The construct pGEM-hTRT is created as initial construct for the constructs pGEM-hTRT(Ka), pGEM-hTRT(Kb) and pGEM-hTRT(Kc). pGEM-hTRT(Ka) pGEM-hTRT(Kb) and pGEM-hTRT(Kc) differ from pGEM-hTRT and from one another by a randomized exchange of sequence of about 20 base pairs (bp). The constructs are used for in vitro transcription with Sp6 RNA polymerase of the standard RNA: hTRT(Ka), hTRT(Kb) and hTRT(Kc). To form the construct pGEM-hTRT, the cDNA of the catalytic subunit of human telomerase (Fig. 1 and SEQ ID. NO. 10) is cloned, for example, into the NotI and HindIII cleavage sites of pGEM-13Zf(+). This is achieved by carrying out an RT-PCR with these cleavage-site-containing oligonucleotide primers, which are derived from the sequence hTRT (Fig. 1 and SEQ ID. NO. 10), on the previously isolated RNA from tumor cells or lines under the conditions described above. Thus, it is possible, for example, to amplify the full-length hTRT with given cleavage sites, or a shorter fragment. After a restriction hydrolysis with specific restriction enzymes, for example NotI and HindIII, the fragment formed is cloned into the corresponding cleavage sites (for example position 12 or 38) of pGEM-13Zf(+) and the construct pGEM-hTRT is created. pGEM-hTRT(Ka) is constructed by replacing an about 20bp sequence in the construct pGEM-hTRT is created. pGEM-hTRT(Ka) is constructed by replacing an about 20bp sequence in the construct pGEM-hTRT by an about 20bp cassette. This replacement is carried out by recombinant PCR and is a modification of the method described by Higuchi et al. [Higuchi, R. (1988). Nucleic Acid Res 16: 7351-7367; Higuchi, R. (1990). M. Innis A. et al. Eds. San Diego, New York, Berkley, Boston, London, Sydney, Tokyo, Toronto, Academic Press, Inc. 177-183]. In a first step, two independent PCR reactions are carried out on pGEM-hTRT: the amplification product from the 1st PCR gives the 5′ fragment and is digested with suitable restriction enzymes to give a 5′ fragment. The amplification product from the

2nd PCR reaction gives the 3' fragment and is hydrolyzed with suitable restriction enzymes to give a 3' fragment. Using T4 ligase, the cleavage sites of the 5' and 3' fragments are connected to give a fragment which is cloned into the corresponding cleavage sites of pGEM-13Zf(+), to create the construct pGEM-hTRT(Ka). pGEM-hTRT(Kb) and pGEM-hTRT(Kc) are constructed by replacing the about 20 bp sequence, created above, in the construct pGEM-hTRT(Ka), in each case with a randomized sequence of about 20bp. RNA can then be produced in vitro from pGEM-hTRT(Ka), pGEM-hTRT(Kb) and pGEM-hTRT(Kc) with Sp6 RNA polymerase. The specific RNAs can then be detected with oligonucleotides O(Ka), O(Kb), O(Kc) and W(wt), which are complementary to the abovementioned about 20bp replacement sequences and to the wild-type sequence (wt), respectively. The further processing of the RNA, such as DNAse digestion, purification and calibration, is carried out by standard methods.

Please amend claims 1, 20, 24, 29 and 34 as follows:

1. (Amended twice) A method for the quantification of tumor cells in a body fluid, comprising:

(a) concentrating or depleting tumor cells in a sample of a body fluid; [and]

(b) specifically amplifying, from the tumor cells, mRNA coding for the catalytic subunit of telomerase; and

(c) quantitatively determining the amount of amplified nucleic acid, thereby quantifying tumor cells in a body fluid.

20. (Amended twice) The method of Claim 1, wherein [for concentrating the tumor cells,] step (a) comprises:

(i) covering a cell separation medium with a layer of the body fluid; and

(ii) centrifuging the cell separation medium covered with the body fluid so that the tumor cells are collected at the interface of the cell separation medium and the supernatant body fluid [centrifuged].

24. (Amended twice) The method of Claim 20, wherein the body fluid is blood and prior to applying the body fluid sample to the cell separation medium, the body fluid [it] is mixed with one or more substances that prevent aggregation of platelets to tumor cells, and/or prior to applying the body fluid sample to the cell separation medium, the body fluid [it] is freed of substances that promote aggregation of platelets to tumor cells.

29. (Amended twice) The method of Claim 20, wherein after centrifugation and before collecting the tumor-cell-enriched interface [interphase], the centrifugation vessel is removed and cooled [intensively] to prevent mixing of the cells in the different layers.

34. (Amended twice) The method of Claim 20, wherein a dye is added to color the cell separation medium [contains a dye], whereby the color of the cell separation medium is distinguishable from that of the supernatant body fluid.